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Hepatitis C virus present in the sera of infected patients interferes with the autophagic process of monocytes impairing their *in-vitro* differentiation into dendritic cells

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ABSTRACT

Autophagy has a pivotal role in the *in-vitro* monocyte differentiation into macrophages and dendritic cells (DCs), the most powerful antigen presenting cells (APC) with the unique capacity to initiate an adaptive immune response. Autophagy is also a mechanism by which these cells of innate immunity may degrade intracellular pathogens and mediate the antigen processing and presentation, essential to clear an infection. For these reasons, pathogens have learned how to manipulate autophagy for their own survival. In this study we found that hepatitis C virus (HCV), derived from sera of infected patients, blocked the autophagic process in differentiating monocytes, seen as LC3 II and p62 expression levels. The suppression of autophagy correlated with a reduction of cathepsins D, B and proteolytic activity, and resulted in impairment of monocyte differentiation into DCs, as indicated by the reduction of CD1a acquirement. These data suggest that the block of autophagy might be one of the underlying mechanisms of the HCV-mediated immune subversion that frequently leads to viral persistence and chronic hepatitis.

1. Introduction

Human hepatitis C virus (HCV) is a positive-stranded RNA virus with size of 50 to 80 nm in diameter, belonging to the *Flaviviridae* family that exists in six different genotypes. It is an important cause of morbidity and mortality worldwide, since it has been reported that approximately 170 million of people are infected. No vaccinations are at the moment available and the current therapies are not always efficient to clear the infection, particularly in patients harboring HCV genotype 1, which is the most common genotype in Western Europe [1]. Recently, direct acting anti-viral drugs, HCV specific protease inhibitors, have been discovered and shown to be much more effective in the eradication of HCV infection, although they come at a significant cost. HCV infects preferentially hepatocytes, causing acute and chronic hepatitis, although it has been reported that also B and T cells, monocytes, macrophages, macrophage-like Kuppfer cells and dendritic cells can be infected *in-vivo* and *in-vitro* [2–4]. The viral persistence in the infected host suggests that HCV is able to manipulate the immune system in order to escape from its control [5–7]; however the underlying molecular mechanisms are not completely elucidated yet. Progress in understanding HCV biology and the virus–host interaction has remained challenging due to lack of an efficient cell culture system for viral growth. The majority of research on this virus, indeed, utilizes sub-genomic or full-length replicon models that replicate in Huh7 hepatoma cell line or in their more permissive sublines, instead of natural virus [8,9].

Autophagy, a cellular degradative process, aimed at the removal of long lived/misfolded proteins as well as damaged organelles, plays an essential role in regulating cell survival and differentiation [10]. It is characterized by the presence of autophagosomes, whose formation can be monitored by many means such as direct electron microscopy observations as well as western blot analysis, by which the appearance of the lipidated form of microtubule-associated protein light chain 3 (LC3II) can be detected [11]. Autophagy represents a cellular strategy that helps cells to survive in stressful conditions, such as during nutrient shortage or in the course of chemotherapeutic treatments [12–14]. Moreover, in the cells of the immune system, autophagy represents a

Abbreviations: Baf, Bafilomycin; Cleaved PARP, Poly-ADP-ribose polymerase p85 fragment; DCs, Dendritic cells; HCV, Hepatitis C virus; LC3, Microtubule-associated protein 1 light chain 3; Rapa, Rapamycin

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route by which intracellular pathogens are degraded [15], for that reason that pathogens manipulate it in order to escape from the immune control [16–18]. More recently, it has been reported that autophagy, induced by GM-CSF in *in-vitro* differentiating monocytes, drives their differentiation into DCs [19].

Previous studies, utilizing replicon models, have addressed the impact of HCV on autophagy. They have shown that HCV induces an incomplete autophagy in Huh7.5 cells, due to the lack of fusion of the autophagosomes with the lysosomes or, as reported in another study, that HCV genotype 1b, but not genotype 2a, induces an incomplete autophagy in Huh7 cells, by altering the lysosomal compartment [20,21].

In this study, we investigated whether HCV derived from sera of infected patients would influence the autophagic process of monocytes differentiating into DCs. We thought that the natural HCV from infected patients might represent a useful biological system to mimic the virus–host interaction in a way that it is more similar to the natural infection. Replicon HCV models, indeed, seem to have different biological behavior, e.g. they do not generally infect peripheral blood mononuclear cells while HCV isolated from sera of infected patients is able to do so [3,22]. We focused our study on genotype 1 HCV because it is characterized by a lower response to conventional therapy, leading more frequently to chronic infection, and also because this genotype has been shown to have a higher impact on autophagy [20]. We found that HCV derived from sera of infected patients mediated an autophagic block in differentiating monocytes that correlated with reduced expression level of cathepsins, also accompanied by reduced lysosomal proteolitic activity. Cathepsins are the main class of lysosomal proteases that play a fundamental role in the degradation of the autophagic cargo [23]. They are subdivided into three subgroups, according to the aminoacids of their active sites that confer catalytic activity: that is, cysteine (cathepsins B, C, F, H, K, L, N, O, S, T, U, W, and X), aspartyl (cathepsins D and E), and serine cathepsins (cathepsins A and G).

More importantly, we found that the autophagic block, mediated by HCV-positive sera, resulted in the inhibition of monocyte differentiation into DCs, compared to efficient DC differentiation achieved culturing the same cells with sera derived from healthy donors that did not interfere with the autophagic process.

DCs, essential for the activation of T cells, are fewer and dysfunctional, in the course of HCV chronic infection [24]. On the other hand, in the low percent of patients that clear the infection, the major role is played by a robust T cell response, which is strictly dependent on DC activity [25,26].

Therefore our results suggest that the impairment of the autophagic process in differentiating monocytes could be one of the mechanisms through which HCV leads to the inhibition of DC formation, circumvents its lysosomal degradation and consequently alters the DC-mediated viral antigen presentation to the T cells, leading to viral persistence, chronic hepatitis and eventually to hepatocarcinoma.

2. Materials and methods

2.1. Human sera

The experiments were conducted with the understanding and the consent of each participant, and the responsible ethical committee of "Policlinico Umberto I" has approved the experiments. Human sera were obtained from healthy donors (HCV, HIV and HBsAg-negative) or from HCV-infected patients (HIV and HBsAg-negative), in which the viral load was measured by VERSANT HCV RNA 3.0 Assay (Siemens Health Care, Australia; cat. no. 02553870). The limits of detection (LOD) and quantification (LOQ) were 15 HCV RNA IU/ml according to the assay package inserts. Valid results were reported quantitatively (IU/ml), as "positive < LOQ" (if the value obtained was under the LOQ), or as "target not detectable".

Genotyping was performed with the Siemens Versant HCV Amplification 2.0Kit (LIPA).

Sera from 3 patients, which were anti-IgG HCV positive, as measured by ELISA (ADVIA Centaur, Siemens Health Care, Australia; cat. no. 03438099) and who had high levels HCV RNA IU/ml of circulating HCVRNA, (>2 × 106), genotype 1, were used throughout the study. A human HCV core-positive serum tested by western blot (Inno-LIA, Innogenetics, Belgium, Europe; cat. no. 80538) was used to detect HCV core protein.

In some experiments, the HCV-positive sera were ultrafiltered with 30 k Vivaspin columns (Sartorius, Goettingen, Germany, Europe cat. No. VS01S1). This ultrafiltration process, validated for removal of a variety of enveloped and non-enveloped viruses with size from 70 nm to 30 nm, was used to remove HCV particles.

2.2. Antibodies and reagents

The following antibodies were used: mouse monoclonal anti-Cd1a (BD Pharmingen, San Jose, California, USA; cat. no. 558703), rabbit polyclonal anti-LC3I/II (Novus Biologicals, Cambridge, UK; cat. no. NB100-2220SS), mouse monoclonal anti-p62 (BD Transduction Laboratories, San Jose, California, USA; cat. no. 6108333), goat polyclonal anti-cathepsin D (Santa Cruz Biotechnology, Europe; cat. no. sc-6487) and mouse monoclonal anti cathepsin S (Santa Cruz Biotechnology, Europe; cat. no. sc-271573), mouse monoclonal anti-cathepsin B (Abcam, Cambridge, MA, USA; cat. no. ab58802), rabbit polyclonal anti-cleaved PARP (poly-ADP-ribose polymerase p85 fragment) (Promega, Madison, WI, USA; cat. no. G7341), mouse monoclonal anti-b actin (Sigma, St. Louis, USA; cat. no. A2228) and mouse monoclonal anti-tubulin (Santa Cruz Biotechnology, Europe; cat. no. sc-8035).

DQ Red BSA (Molecular Probes) was purchased from Life Technologies (Life Technologies, Monza, Italy, Europe; cat. no. D-12051); bafilomycin A1 (20 nM) (Sigma, St. Louis, USA; cat. no B1793) and rapamycin (1 mM) (Sigma, St. Louis, USA; cat. no. R8781) were purchased from Sigma. Cathepsin D inhibitor (pepstatin A) was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Europe; cat. no. sc-43036) and cathepsin B inhibitor (Inhibitor III) was purchased from Calbiochem (Calbiochem, MA, USA; cat. no. 219419).

2.3. Generation of monocyte-derived DC

To generate monocyte-derived DC, human peripheral blood mononuclear cells, obtained from healthy donors were isolated by Ficoll-Paque gradient centrifugation (Pharmacia, Uppsala, Sweden) from buffy coats. CD14+ monocytes were positively selected using anti-CD14 MAb-conjugated magnetic microbeads (Miltenyi Biotec, Auburn, CA, USA; cat. no. 130-050-201). Purified monocytes (more than 95% CD14+, as evaluated by FACS analysis) were cultured at a density of 1 × 106 cells/3 ml in 12-well plates for 6 days in the differentiating cocktail: RPMI 1640 (Sigma, St. Louis, MO, USA; cat. no. R0883) containing 10% FCS (Euroclone, Milan, Italy; cat. no. ECLS0180L, 2 mM L-glutamine, 100 U/ml penicillin G, 100 mg/ml streptomycin (Gibco, Carlsbad, CA, USA; cat. no. 10378-016) and recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF) plus interleukin 4 (IL-4) (50 ng/ml and 20 ng/ml respectively, (Miltenyi Biotec, Auburn, CA, USA; cat. no. 130-095-372 and cat.no. 130-093-917). Monocytes were exposed to sera of HCV-infected patients or healthy donors for 60 min at 370C and then cultured in the differentiation cocktail in the presence of 20% of the same sera. In some experiments monocytes were differentiated in the presence of bafilomycin A1 (20 nM) and rapamycin (1 μM) or cathepsin inhibitors pepstatin A (10 μM) and inhibitor III (20 μM).

2.4. Analysis of DC phenotype by flow cytometry (FACS analysis)

The phenotype of DCs was monitored by staining with phycoeritrin (PE)-conjugated anti CD1a for 30 min at 4 °C and analyzed by a cytofluorimeter EPICS XL Coulter (Hialeah, FL). DCs were gated

according to their FSC and SSC properties. Appropriate isotype controls were included and 5.000 viable DCs were acquired for each sample.

2.5. Western blot analysis

Monocytes differentiated in the presence of HCV-containing sera or healthy donor sera (10 × 105) were lysed in a modified RIPA buffer containing 150 mM NaCl, 1% NP-40, 50 mM Tris–HCl (pH 8), 0.5% deoxycholic acid, 0.1% SDS, 1% Triton X-100, protease and phosphatase inhibitors. The lysates were prepared according to the manufacturer's instructions (Life Technologies), subjected to electrophoresis on 4–12% NuPage Bis–Tris gels (Life Technologies, Carlsbad, CA; cod. no. NO0322BOX) and transferred to PVDF membranes (Millipore Corporation, Billerica, MA, USA; cat. no. IPVH00010). The membranes were blocked for 1 h in phosphate buffered saline (PBS) (Sigma, St. Louis, USA; cat. no. D8537), blocking solution, containing 3% bovine serum albumine (BSA) (Sigma, St. Louis, USA; cat. no. A4503), and 0.1% Tween 20 (Sigma, St. Louis, USA; cat. no. P5927) and then incubated with a primary antibody overnight at 4 °C. The membranes were washed 5 min for three times in the washing solution (PBS and 0.1% Tween 20) and incubated for 45 min with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Europe; cat. no. sc-2004, sc-2768, sc-2005). The membranes were washed as described before and the blots were developed using ECL blotting substrate (Thermo Scientific, Rockford, USA; cat. no. 32209).

2.6. Red-BSA assay

Monocytes differentiated for 24 h in the presence of healthy donor or HCV-infected patients were treated with DQTM Red BSA (DQ-BSA) (Molecular Probes) (Life Technologies, Monza, Italy, Europe; cat. no. D-12051) (20 mg/ml) for 15 min at 370C, washed twice and kept at 370C for 2 h. Cells were then analyzed using an Apotome Axio Observer Z1 inverted microscope (Zeiss, Oberkochen, Germany), equipped with an AxioCam MRM Rev.3 at 40× magnification.

2.7. Cell viability analysis

Monocytes, differentiated for 5 days in the presence of HCV-negative or HCV-positive sera, were collected and counted by trypan-blue exclusion assay using a hemocytometer. Each experiment was performed in triplicate.

2.8. Statistical analysis

All experimental results, unless differently indicated, were expressed as the arithmetic mean and standard deviation (SD) of measurements. Student's *t*-test was used for statistical significance of the differences between treatment groups. Statistical analysis was performed using analysis of variance at 5% ($p < 0.05$) or 1% ($p < 0.01$).

3. Results

3.1. HCV-positive sera block autophagy during monocyte differentiation

CD14+ monocytes, isolated from healthy donors, differentiate *in-vitro* into DCs when cultured in the presence of GM-CSF and IL-4, for 5 days [27]. It has been reported that GM-CSF, contained in the differentiation cocktail, induces autophagy and that the autophagic process is essential for the *in-vitro* monocyte differentiation [19]. Here we first investigated whether the HCV-positive sera could influence autophagy in monocytes differentiating into DCs. At this aim, we exposed human monocytes to sera derived from HCV-infected patients or from healthy donors (see Materials and methods) and then cultured them in complete medium, containing GM-CSF and IL-4, in the presence of 20% of the same HCV-positive or HCV-negative serum. After 24 h or after 5 days of *in-vitro* differentiation, the two main autophagic markers, LC3 I/II and p62, were analyzed by western blot. We observed an increase of LC3 II level in monocytes exposed to HCV-containing serum in comparison to the same cells exposed to healthy donor serum (Fig. 1A and B), indicating an impact of HCV-positive serum on monocytes' autophagy. Since the LC3 II increase, by itself, does not discriminate between autophagic induction and/or autophagic blockage at the late steps, we analyzed p62, which is a read out of the bona fide autophagic process. The results show a strong accumulation of p62 protein levels in the monocytes cultured in the presence of HCV-positive serum for 24 h and after 5 days of *in-vitro* culture (Fig. 1A and B), in comparison to the same cells exposed to the healthy donor-serum, suggesting that a block of the autophagic flux was occurring in these cells. After 24 h of viral-exposure, HCV core protein was transiently detected, by western blot analysis, in differentiating monocytes exposed to HCV-positive serum and it was no more visible after 5 days culture (Fig. 1 A and B). A possible explanation of HCV core disappearance might be that HCV binds or enters in differentiating monocytes but it does not efficiently replicate in them, as occurs in differentiated DCs or that the HCV-infected monocytes do not survive after 5 days of culture (see below), according to previously reported studies [28,29]. Then, to clarify whether the effects on autophagy were mediated by the virus itself or by the molecules, such as cytokines, possibly present in HCV-positive sera, we ultrafiltered the HCV-positive sera through 30 k membrane columns (HCV + filtered), to remove the viral particles. The results obtained show that the increase of LC3 II level in monocytes exposed to HCV-containing serum was efficiently reduced by removing the viral particles from the sera (Fig. 1C), highlighting the role of HCV itself in mediating the autophagy block in differentiating monocytes. Finally, to confirm that autophagy was blocked at late steps by HCV-containing serum, we compared its effect with the effect mediated by bafilomycin (baf),

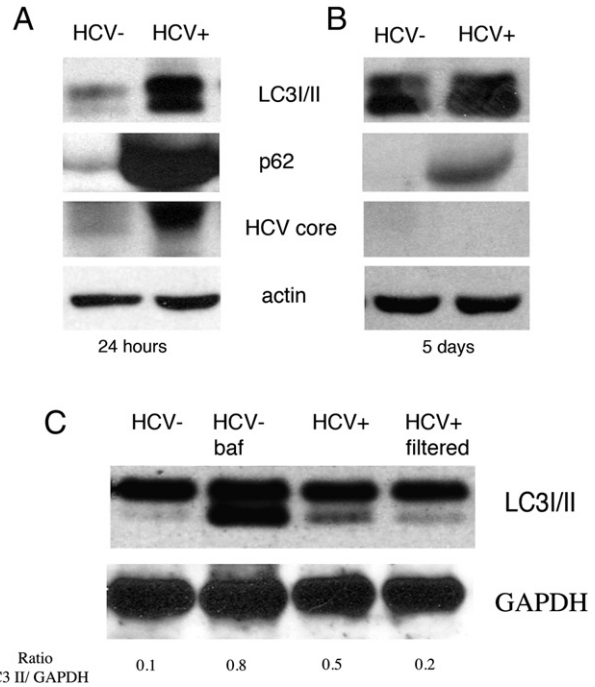

Fig. 1. Human HCV-positive sera block autophagy in differentiating monocytes. A) Expression levels of LC3I/II, p62 and HCV-core protein were analyzed by western blot of equal amount of total cell extracts of monocytes undergoing A) 24 h and B) 5 days of *in-vitro* differentiation, induced by GM-CSF and IL-4 in the presence of healthy donor (HCV−) or HCV-positive sera (HCV+). Anti-actin was used as protein loading control. C) Western blot analysis of LC3I/II expression level of monocytes cultured in the presence of healthy donor (HCV−), HCV-positive (HCV+), HCV-positive ultrafiltered (HCV filtered) sera and bafilomycin (baf) is reported. GAPDH was used as protein loading control. A densitometric analysis based on the ratio LC3 II/GAPDH is reported and a representative experiment out of three is shown.

which is a well known blocker of the final degradative steps of autophagy. The results obtained showed that LC3 II level increased both in monocytes cultured in the presence of baf or in the presence of HCV-positive sera (Fig. 1C).

3.2. HCV reduces the expression level of cathepsins B and D in differentiating monocytes

We next investigated which molecular mechanism/s could underlie the autophagic block at late steps mediated by HCV containing sera in differentiating monocytes. We analyzed the expression level of cathepsins since these molecules play a major role in the degradation of the autophagic cargo and have been previously shown to influence the bona fide outcome of the autophagic process [23]. Moreover, it has been previously reported that HCV reduces the level of cathepsin B or S in other cell types [20,30]. As shown in Fig. 2A, the expression level of cysteine cathepsin B was strongly reduced in differentiating monocytes exposed to HCV-containing serum while cathepsin S was slightly affected (Fig. 2A). Cathepsin B plays an important role in the degradative steps of autophagy, while cathepsin S is involved in the MHC presentation, one of the most important functions of these cells [31]. Finally we evaluated the expression level of the aspartyl cathepsin D, which, together with cathepsin B, represents the most important cathepsin involved in the degradative process of autophagy in monocytes. These cells, indeed, do not express cathepsin L, the other protease with a major role in such process [32]. We found that cathepsin D expression was also strongly reduced by serum of HCV-infected patients in differentiating monocytes, after 24 h of *in-vitro* culture (Fig. 2B). To evaluate whether the reduced expression level of cathepsins in monocytes cultured in the presence of HCV-containing sera was also accompanied by a reduced lysosomal proteolytic activity, we treated differentiating monocytes with DQTM Red (DQ-BSA). This molecule is a derivative of bovine serum albumin that is labeled with self-quenched red fluorescent dye and can be dequenched by proteases, such as cathepsins, present in the lysosomes [23,33]. As in other studies, where the cathepsin content was reduced [23], we found that the red staining of DQ-BSA was less bright in monocytes cultured in the presence of HCV-containing sera for 24 h, in comparison to those cultured in the presence of healthy donors' sera (Fig. 2C), indicating a reduced proteolytic activity, concomitantly with the reduced cathepsin expression observed by western blot analysis (Fig. 2A and B). Altogether, these data indicate that HCV-containing sera reduced the expression level of cathepsins and proteolytic activity in differentiating monocytes.

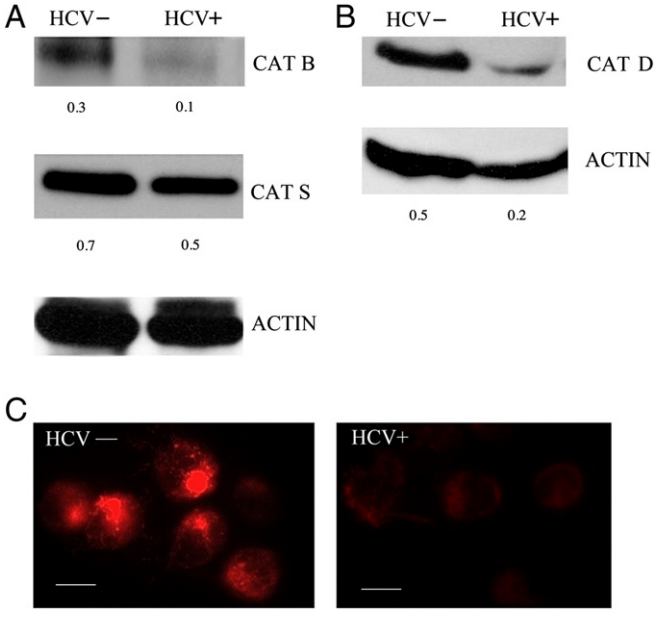

Fig. 2. Human HCV-positive sera reduce cathepsin expression in differentiating monocytes. A) Western blot analyses showing the expression levels of cathepsin B, S and B) cathepsin D in monocytes differentiated for 24 h in the presence of HCV-negative (HCV−) or HCV-positive (HCV+) sera. β-actin was used as protein loading control. A densitometric analysis based on the ratio cathepsins/actin is reported and a representative experiment out of three is shown. C) Fluorescence analysis of human primary monocytes differentiated for 24 h in the presence of HCV-negative (HCV−) or HCV-positive (HCV+) sera treated with DQ-BSA to evaluate the proteolitic activity. A representative experiment out of three is shown. Bars = 10 μm.

3.3. The HCV-mediated cathepsin reduction results in an impairment of monocyte differentiation into DCs

We showed above that HCV-containing sera impaired autophagy and lysosomal activity during monocyte differentiation. Since autophagy, induced in monocytes by GM-CSF contained in the differentiating cocktail, seems to be essential for their *in-vitro* differentiation into DCs [19], we investigated whether the autophagic block, induced in monocytes by sera of HCV infected patients, could impair their differentiation into DCs. At this aim, we analyzed the surface expression of CD1a, a marker typically expressed by differentiated DCs. As control, to confirm that autophagy modulation can affect the monocyte differentiation, we included the autophagic blocker bafilomycin A1 (baf) and the autophagy inducer, rapamycin (rapa). As shown in Fig. 3A, after 3 days of culture, the expression level of CD1a was reduced in DCs exposed to HCV-containing serum (HCV+) in comparison to DCs differentiated in the presence of differentiation cocktail only (control). Similar results were obtained in the presence of the autophagy inhibitor baf and CD1a expression was slightly affected in DCs exposed to healthy donor serum (HCV−) (Fig. 3A). On the other hand, in the presence of rapa, after only 3 days of *in-vitro* culture, CD1a expression reached the level of completely differentiated cells, which is usually achieved by DCs after 5 days (Fig. 3A). These data are in agreement with previous studies indicating that the autophagic process is essential for monocyte differentiation and suggest that its block by HCV-containing sera, similarly to pharmacological autophagy inhibitors, leads to a reduction of DC formation. After 5 days of *in-vitro* culture, when the differentiating process is complete, CD1a surface expression was again evaluated in DCs. We found that CD1a expression was still reduced in DCs cultured in the presence of HCV-positive serum or of the autophagic blocker baf, compared to the healthy donor serum (HCV−), either in terms of percentage of positive cells or mean fluorescence intensity (MFI) (Fig. 3B and C). Of note, when the viral particles were removed through ultrafiltration, HCV-depleted serum (HCV filtered) no longer affected monocyte differentiation, in a way similar to the healthy donor serum (Fig. 3B and C) and in agreement with the little influence on autophagy seen in Fig. 1C. These data highlight the role of HCV particles in both the impairment of monocyte differentiation and in the block of the monocyte autophagy.

Since the autophagic block and the impairment of DC formation mediated by HCV may occur through the reduction of cathepsin B and D expression, we next analyzed the effect of both cathepsin D and B inhibitors (pepstatin A and inhibitor III, respectively) on monocyte differentiation into DCs. The results indicate that cathepsin B and D inhibitors impaired DC differentiation (Fig. 4A and B), suggesting that cathepsins are required for monocyte differentiation. Cathepsin reduction by HCV-positive sera could be the mechanism underlying the impairment of monocyte differentiation.

3.4. The block of autophagy by HCV-positive sera reduces cell survival in differentiating monocytes

To evaluate if the autophagic block caused by HCV-positive sera would also affect cell survival in differentiating monocytes, we performed a trypan-blue exclusion viability assay. The results, shown in Fig. 5A, indicate that about 90% of cell survival observed in the presence of healthy donor sera significantly dropped down to 50% in the presence

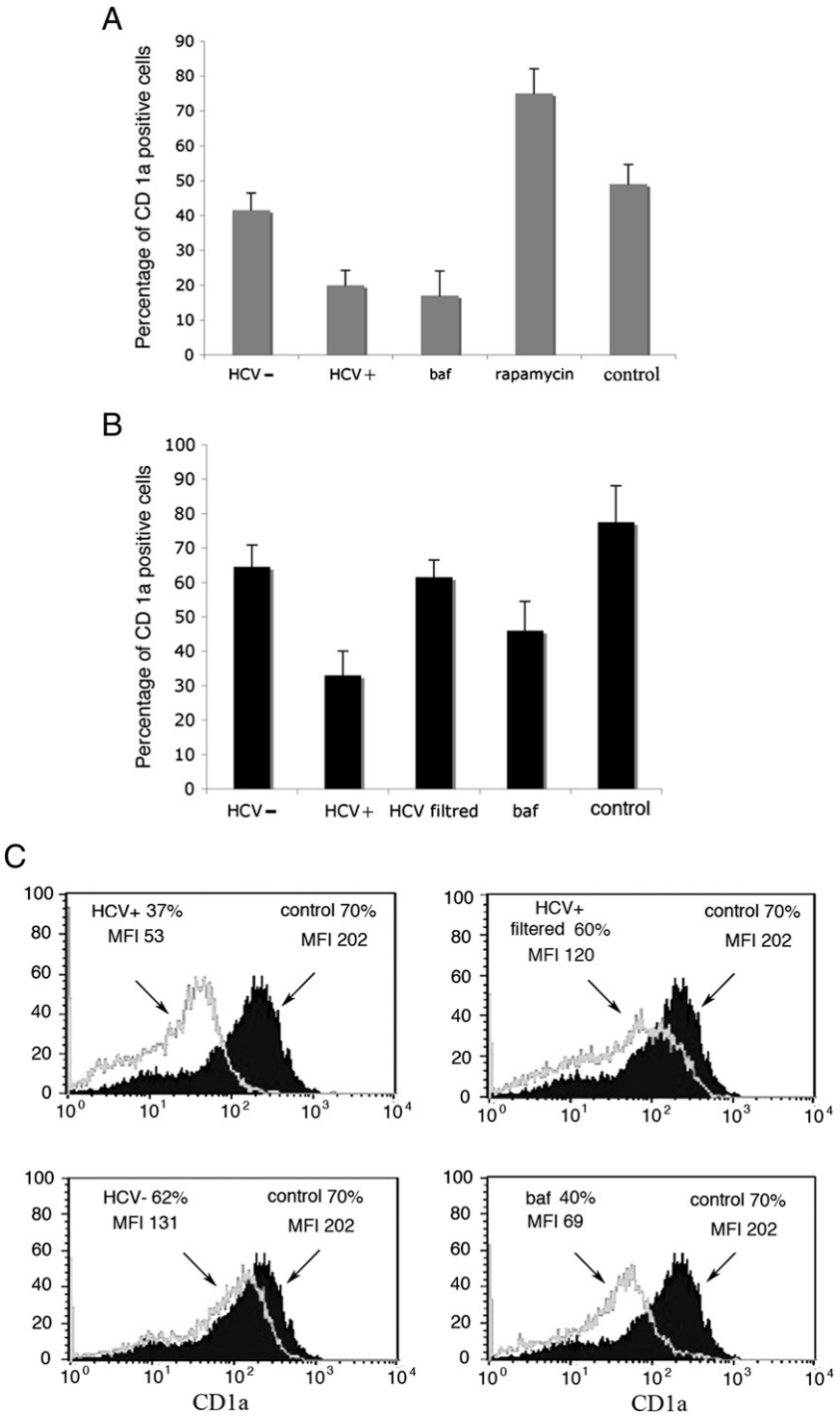

Fig. 3. Human HCV-positive sera impair monocyte differentiation into DCs. A) FACS analysis showing the CD1a expression in human primary monocytes differentiated for 3 days in the differentiation cocktail containing GM-CSF and IL-4 in the absence (control) or in the presence of HCV-negative (HCV−) or HCV-positive sera (HCV+) or ultrafiltered HCV-positive serum (HCV filtered) or bafilomycin (baf) or rapamycin (rapa). Histograms represent the mean ± SD of three independent experiments. B) FACS analysis showing the CD1a expression in DCs differentiated for 5 days in the differentiation cocktail in the absence (control) or in the presence of HCV-negative (HCV−) or HCV-positive sera (HCV+) or ultrafiltered HCV-positive (HCV filtered) sera or bafilomycin (baf). The results shown are percentage of CD1a positive cells ± SD of three independent experiments. C) FACS analysis, as staining profiles, of a representative experiment showing the percentage of CD1a and mean fluorescence intensity (MFI) of monocytes differentiated in the presence of HCV-negative (HCV−) or HCV-positive (HCV+) or ultrafiltered HCV-positive (HCV filtered) sera or bafilomycin (baf) over control differentiated cells.

of HCV-containing sera, both in comparison with control monocytes differentiated in the GM-CSF and IL-4 differentiation cocktail. To further demonstrate the induction of cell death by HCV-containing sera, PARP cleavage was evaluated by western blot analysis. The results show a strong PARP cleavage in monocytes differentiated in the presence of HCV-positive sera compared to monocytes cultured in the presence of healthy donor sera (Fig. 5B). These data suggest that the autophagic block mediated by HCV, aside from hampering monocyte differentiation, also induced cell death in these cells.

4. Discussion

HCV infection becomes chronic in about 80% of patients, indicating that the virus is able to circumvent the immune response [24]. DCs

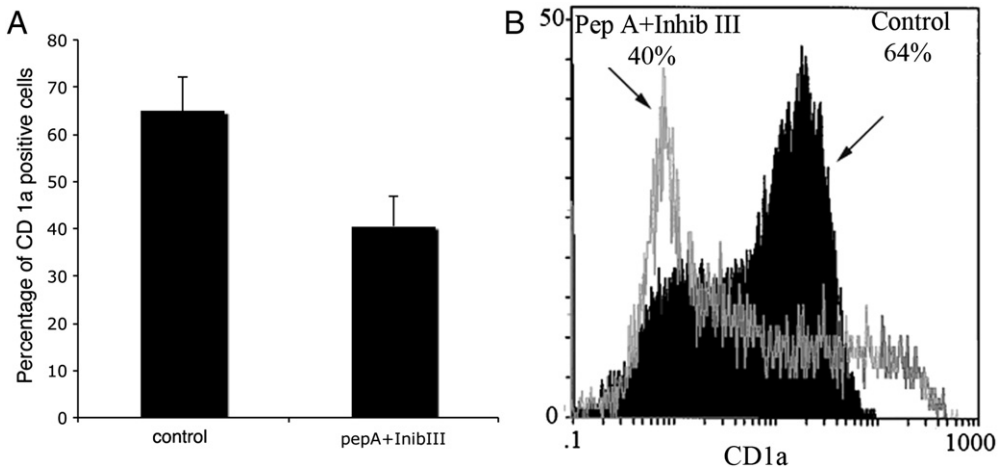

Fig. 4. Cathepsin D and B inhibition impairs monocyte differentiation into DCs. FACS analysis of CD1a expression in DCs differentiated for 5 days in the presence or in the absence of cathepsin D (pepstatin A) and cathepsin B inhibitor (inhibitor III). A) Mean ± SD of three independent experiments is reported and B) data from a representative experiment, as staining profiles, showing CD1a expression of DCs differentiated in the presence of the combination of pepstatin A and inhibitor III over control differentiated cells.

have a central role in both innate and adaptive immunity, being able to sense pathogens, phagocyte them and present their processed peptides to T cells, initiating a specific immune response. The DC functional impairment, frequently observed in patients affected by chronic hepatitis C, could be a possible underlying mechanism of such immune dysfunction [24]. Moreover, it has been shown that DCs derived from patients with HCV persistent infection stimulate the proliferation of regulatory T cells (Tregs), which further impairs the HCV-specific T cell response [34] and that HCV core protein may play a major role in the development of IL-10-producing Tregs [35]. The interference with DC development and functions, *in-vivo* and *in-vitro*, represents a common strategy utilized by viruses, belonging to different families, to escape from immune control [36–38]. *In-vitro* studies on HCV have reported that core and NS3 recombinant proteins are able to inhibit the *in-vitro* differentiation of monocytes into DCs and also that HCV replicon model, JFH-1, interferes with DC maturation [39,40]. However extensive immunological *in-vitro* studies with whole virus have been hampered by the absence of a cell culture system for HCV and most of the studies use the replicon viral models that are not generally able to infect immune cells [22]. *In-vitro* monocyte infection can be achieved with the virus isolated from plasma or serum of HCV-infected patients and it is known that monocytes [3,41], DC macrophages, and B and T lymphocytes harbor HCV infection *in-vivo* [8]. In this study we used natural HCV from sera of HCV-infected patients to reproduce more closely the interplay between monocytes and the virus that occurs *in-vivo*, in the course of natural infection. We found that, in the presence of HCV-containing sera, the autophagic flux, essential for monocyte differentiation, was blocked and that this blockage resulted in an impairment of the monocyte differentiation into DCs. Similar effects were obtained by using baf, a well known autophagic blocker, confirming that a complete autophagic process is required for DC formation. Of note, when sera of HCV-infected patients underwent ultrafiltration to remove HCV particles, DC differentiation was no longer affected, indicating that HCV present in infected sera was playing the major role in inhibiting DC formation. Interestingly, we found that the differentiating monocytes exposed to HCV-containing sera, transiently expressed HCV core protein which, according to a previous study [35], could play an important role in the immune suppression. Since it has been reported that monocytes derived from HCV-positive patients remain HCV-infected only for 1 day in culture because cell death occurs selectively in the infected monocytes [1], we speculate that this could be the reason for the disappearance of HCV core expression in differentiating monocytes after 5 days of *in-vitro* culture (Fig. 1B) and for the increased cell death observed in these cells (Fig. 5) [28]. HCV replicon models and hepatoma cell lines have been previously used to investigate the role of autophagy in the pathogenesis of HCV-induced hepatitis or in type I

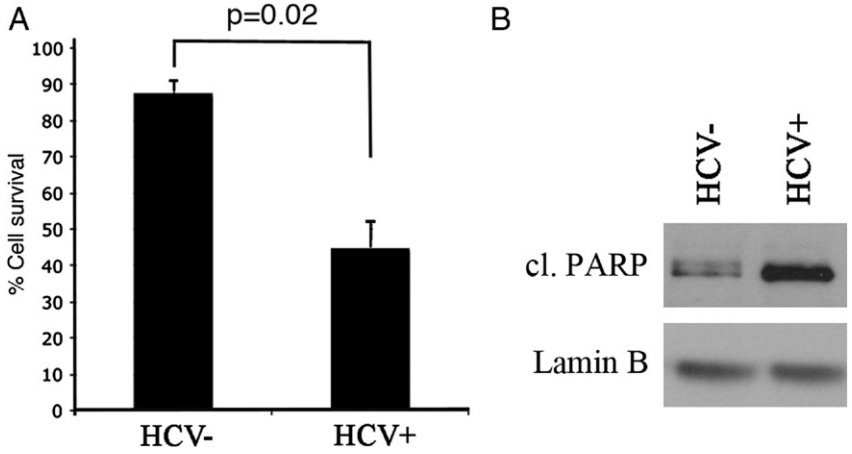

Fig. 5. Human HCV-positive sera reduce the human primary monocytes survival. A) DCs, differentiated for 5 days with GM-CSF and IL-4 in the presence of healthy donor (HCV−) or HCV-positive (HCV+) sera, were counted by trypan blue exclusion in comparison to the control DCs differentiated in the cocktail containing GM-CSF and IL-4 in the absence of sera. Mean ± SD of cell survival and *p* value of three different experiments is reported. B) Western blot analysis with anti-cleaved PARP (poly-ADP-ribose polymerase) p85 fragment antibody of DCs differentiated in the presence of healthy donor (HCV−) or HCV-positive (HCV+) sera. A representative experiment is shown. Lamin B was used as protein loading control.

IFN production [21,42]. In this study, we found that the HCV-containing sera blocked the autophagic flux in differentiating monocytes, in correlation with a reduction of cathepsin D and B expression and with a decreased proteolytic activity.

Cathepsins B and D are the cysteine and aspartyl cathepsins fundamental for the degradation of the autophagic cargo in the lysosomal compartment [23], while the cysteine cathepsin S, also partially reduced by sera of HCV-infected patients, could interfere with the invariant chain (Ii) cleavage, Major Histocompatibility Complex (MHC) class II maturation and surface expression and finally with antigen presentation [31]. In a previous study, it has been reported that HCV, and in particular its core and NS5a proteins, reduced cathepsin S expression and as a consequence the Ii resulted to be more expressed on the cell surface, concomitantly with a reduction of mature MHC class II [30]. Our results suggest that the cathepsin reduction in DCs may represent an important HCV strategy to escape from the immune control. Indeed, by reducing cathepsins B and D, HCV blocked the autophagic process and impaired DC differentiation. The autophagic block could further promote the viral immune escape by interfering with the viral degradation in the lysosomes and MHC class II antigen presentation. The latter could be also impaired by HCV-mediated reduction of cathepsin S. It will be interesting to investigate how HCV-containing human sera would affect autophagy and differentiation of monocytes *in-vivo*, using the recently developed humanized mouse model AFC8-hu HSC/Hep [43]. These mice, containing human immune system and liver cells, could be useful to evaluate the impact of HCV-mediated DC functional impairment on the liver diseases. Moreover it will be interesting to analyze if the recently discovered anti-HCV drugs could influence autophagy in immune as well as in liver cells.

5. Conclusions

In summary, this study showing that HCV blocks autophagy and monocyte differentiation into DC unveils a new strategy utilized by HCV to ensure its persistence in the host and to promote the onset of chronic virus-associated diseases. The understanding of the HCV-mediated effects on the immune system is essential to be able to design new immunotherapies effective against HCV.

Authorship

M.C.: conceived and designed the experiments. M.G., V.L., M.P., L.DR, S.V.: performed the experiments. M.C., G.A., L.F., A.F.: analyzed the data. D.R.: contributed reagents/materials/analysis tools. M.C., A.F.: wrote the paper:

Conflict of interest disclosure

The authors declare no conflict of interest

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